LC-MS^2-Based Dereplication of *Euphorbia* Extracts with Anti-Chikungunya Virus Activity

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Chemical compounds studied in this article

12-*O*-tetradecanoylphorbol-13-acetate (PubChem CID:27924), 12-deoxyphorbol-13-acetate (PubChem CID:45217), phorbol-12,13-didecanoate (PubChem CID:71308620), ingenol-3-mebutate (PubChem CID:6918670), ingenol-3,20-dibenzoate (PubChem: 442043), resiniferatoxin (PubChem CID:5702546)

A B S T R A C T

Recently, diterpenoids from various Euphorbiaceae species, such as phorbol esters, have been shown to be potent inhibitors of chikungunya virus (CHIKV) replication. To discover new natural inhibitors of chikungunya virus (CHIKV) replication, forty-five extracts prepared from various plant parts of 11 Euphorbiaceae species (*Euphorbia* and *Mercuralis* genus) were evaluated for antiviral effect against CHIKV in a virus-cell-based assay. All EtOAc extracts from *Euphorbia* species exhibited potent and selective anti-CHIKV activity, latex extracts proved to be the most potent inhibitors. A LC-MS^2-based dereplication strategy was established for the detection in the active extracts of known substances displaying potent antiviral activity such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (**13**), phorbol-12,13-didecanoate (**11**) and prostratin (**21**), and twenty-four other commercially available diterpenoids of tigliane-, ingenane- and daphnane-type. This approach allowed the identification of three compounds in *Euphorbia* extracts: ingenol-3-mebutate in *E. peplus*, 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate in *E. segetalis* ssp. *pinea* and ingenol-3,20-dibenzoate in *E. peplus* and *E. pithyusa* ssp. *pithyusa*. Known potent inhibitors of CHIKV replication such as phobol esters were not identified in the *Euphorbia* extracts. Thus, the present study suggested that untargeted diterpene esters are responsible for the antiviral properties of the *Euphorbia* extracts.

Keywords:

Euphorbia extracts, Diterpene esters, LC-MS^2, Antiviral activity, Chikungunya virus

1. Introduction

Chikungunya fever is caused by an arthropod-borne virus that is associated with massive epidemics and severe morbidity (virus-induced arthralgia, fever, myalgia and rashes). Worldwide expansion of the mosquito vectors *Aedes aegypti* and *A. albopictus* is responsible for the spread of chikungunya virus (CHIKV) from Africa and the Indian subcontinent to Southeast Asia, around the Indian Ocean, and more recently to the Caribbean islands, Central and South America [1–3]. Currently, no antiviral drugs or vaccines are available for the treatment or prevention of CHIKV infection [4]. Recent scientific reviews have highlighted issues and latest developments in the search for new therapeutic solutions [5,6].

In an effort to identify novel inhibitors of CHIKV replication, Euphorbiaceae species have been selected and investigated by means of bioassay-guided purification, which resulted in the isolation of daphnane- and tigliane-type esters with anti-CHIKV activity [7,8]. In particular, tigliane-type esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (**13**), phorbol-12,13-didecanoate (**11**) and prostratin (**21**) were found to be potent and selective inhibitors of CHIKV and HIV replication *in vitro* [9,10] However, TPA along with other phorbol esters are known to possess pro-inflammatory and tumor-promoting activities [11–14] through broad activation of PKCs (Protein kinase C isoenzymes) [15,16].

The genus *Euphorbia* is the largest genus in the family Euphorbiaceae with over 2.000 species, ranging from prostrate annuals, cactus-like succulents to tall trees [17]. In Europe, this genus is represented by more than hundred species that mainly belong to the subgenus (subg.) *Esula* Pers., which vary from annual herbs to small shrubs growing in the wild in Mediterranean region [18,19]. In Corsica, the *Euphorbia* genus consists of 33 taxa, of which 22 are endemic to Euro-Mediterranean areas [20]. Spurges (*Euphorbia* spp.) produce an irritant milky-white sap (latex), which acts as a chemical defense barrier when the plant is wounded. The toxicity of the latex to the skin, mucosae and eyes has been known since ancient time [21] and is still a common cause of gardening mishaps [22]. According to Greek and Roman literature, medical utilization of spurges included treatment of cancerous conditions, relieve of chronic pain and as drastic purgative [21,23]. In Corsica, an ethnobotanical study revealed that latex of *Euphorbia* spp. was traditionally used as vesicant agent to remove warts [24]. In Sardinia and in Central Italy, ethnobotanical studies reported that *E. characias* and *E. rigida* were used as fish poison to flush out the eels which other while would suffocate [25,26].

Diterpenoids isolated from *Euphorbia* species represent a unique group of structurally diverse compounds that possess remarkable biological activities [27–31], such as potent antiviral activity against human immunodeficiency virus (HIV) for tigliane-[32], ingenane- [28,33,34] and macrocyclic-type esters [35–37], or powerful P-glycoprotein modulation activity for macrocyclic diterpene esters [38,39].

The recent launch of Picato® (ingenol-3-mebutate), a broad PKC modulator isolated from *E. peplus* for the treatment of precancerous skin condition (actinic keratosis), highlights the therapeutic potential of diterpene esters [40]. In addition, EBC-46, a tigliane-type diterpene ester, was able to induce regression and ultimate cure of diverse tumors following a single intra-lesional injection in a pre-clinical model for cancer [41].

Several LC-MS based methods targeting commercial and isolated compounds were developed in order to monitor diterpene esters of tigliane [42,43], ingenane [42,44–46], lathyrane [46–48] and daphnane-types [49,50].

In the present study, 45 extracts from different plant parts of eleven Euphorbiaceae (including ten *Euphorbia* and one *Mercurialis* species) were evaluated for selective inhibition of CHIKV in a virus-cell-based assay. In a dereplication perspective, a targeted LC-MS^2-based method was developed for the detection of 27 diterpene esters possessing antiviral properties (anti-CHIKV and/or anti-HIV) [10], or known to possess PKC modulation ability, pro-inflammatory and tumour-promotion activities. The bioactivity guided-fractionation procedure performed on an EtOAc extract of *E. amygdaloides* ssp. *semiperfoliata* was performed following the work presented herein [51].

2. Experimental

2.1. Plant material

The samples of various Euphorbiaceae were collected on different locations in Corsica Island (France) during July and August 2011 (Table 1). The botanical identification of the species has been established by Louis-Félix Nothias-Scaglia. A voucher specimen for each species has been deposited at the Herbarium of the CPN laboratory at the University of Corsica (Corte).

2.2. Extract preparation

Harvested plants were air-dried for a period of three weeks at ambient temperature. The vegetal parts were powdered using a blade miller (PX-MCF 90D Kinematica). All the samples were extracted with 3 x 100 mL of ethyl acetate (EtOAc) and subsequently, with 3 x 100 mL of methanol (MeOH) using an automatic solvent extractor (ASE 200). For each solvent, a maceration was carried out during 15 min at 40 °C and 34 bar. The solution was evaporated to dryness *in vacuo* yielding dried extract.

The latex were collected into EtOH after making cuts on stalks. A whitish precipitate was removed from the latex by cotton filtration. Filtrates were evaporated to dryness *in vacuo* yielding crude extract. The laths were partitioned by liquid-liquid extraction with EtOAc and water. For some latex, an emulsion was observed during liquid-liquid partition and thus, the solution was evaporated to dryness using a rotary evaporator and solid-liquid extractions were performed using EtOAc. All extracts were stored at 4°C until analysis. Solvents and others chemicals were purchased from VWR (France). The samples used for LC-MS^2 analysis were prepared by dissolving the extracts in MeOH at 2.5 mg/mL, and then filtered on 0.2 µm PTFE filter.

2.3. MS^2 and LC-MS^2 analysis

2.3.1. Chemicals and standard compounds

Solvents and reagents used for sample preparation and chromatography were LC-MS grade: acetonitrile (ACN), methanol (MeOH), and ammonium acetate (NH_4AcO) were obtained from Fisher Scientific (Illkirch, FR). Deionized water was purified by Milli-Q water Millipore (Bedford, USA) purification system. All reference compounds **1**-**24** and **26**-**27** (98% purity by LC) were purchased from Santa Cruz Biotechnology Inc (Heidelberg, Germany) except ingenol-3-angelate **25** (98% purity by LC), which was purchased from Coger SAS (Paris, France). Solutions of reference compounds

were prepared by dissolving each component in MeOH at 1 mg/mL and then filtered on 0.2 µm PTFE filter. Flow injection analysis (FIA) were performed with reference solutions at a concentration of 0.1 mg/mL in 8:2 ACN/H_2O + 0,1 % NH_4AcO.

2.3.2. Analysis by flow injection analysis MS (FIA)

Experiments were performed using a 3200 QTRAP AB Sciex (Framingham, MA, USA) linear triple quadrupole mass spectrometer fitted with ESI Turbo V^{TM} ion source operating in positive mode. High purity nitrogen was used both as nebulizer and turbo gas. The ESI source parameters used for FIA were set as follow: CUR (curtain gas), 10 psi; CAD (collision gas), high; GS1 (nebulizer gas), 20 psi; GS2 (heater gas), 0 psi; IS (ion spray voltage), 5000 V; temperature (150°C). The software used for data acquisition and data analysis was Analyst version 1.5.1 (Framingham, MA, USA).

For each reference compounds, a relevant transition of pseudo-molecular ion was selected using the automated component optimization function of the Analyst software. The instrumental parameters were also optimized in direct infusion (flow rate : 10 µL/min) to achieve maximum signal/noise (S/N).

2.3.3. LC-MS^2 analysis

The LC system consists of a Flexar LC PerkinElmer (Waltham, MA, USA) made up of two Flexar FX-10 LC pump, a Flexar solvent manager, a 275-Flexar autosampler, and a Flexar LC PE200 column oven. LC separations were performed on a 100×2.1 mm i.d., RP 18, 3 μm, LUNA 3U column (Phenomenex) and the column temperature was set at 25°C. The injected sample volume was 10 μL using an injection loop of 15 μL in partial loop mode. The mobile phase consisted in milliQ water (solvent A) and ACN (solvent B) each containing 0.1% (v/v) NH_4AcO buffer. During LC analysis, the flow rate was set at 700 μL/min and equilibration of the column was perform by a 50% A-50% B elution (5 min); elution was carried out with the following steps: 50 % A-50% B for 1 min, followed by a linear gradient of 50-75% B during 16 min; increased from 75% B to 100% in 4 min; and 100% B during 10 min. The ESI source parameters were optimized to achieve maximum detection of diterpene esters and the following parameters were used for LC-MS^n analysis: CUR (25 psi); CAD (high); GS1 (45 psi); GS2 (40 psi); IS (5000 V); temperature (500°C). MS^2 spectra were acquired by an MS^2 scan with the following parameters: Q1 resolution (unit), Q3 resolution (unit); DP (declustering potential) 70 V; EP (entrance potential) 10 V; CE (collision energy) 35 V and CES (± 15 V). To achieve maximum sensitivity, data acquisition was performed by scanning specific precusor-to-product transition of each standard compound in the multiple reaction monitoring (MRM), followed by automatic acquisition of MS^2 spectrum in EPI (Enhanced Product Ion) mode. MS^2-EPI mass

spectra were recorded in the range of *m*/*z* 50-1000 at 4000 Da/s. Retention times, MRM transitions and multiple MS^2 spectra of each standard compound were recorded into Analyst software spectral database. Several mixtures of standard compounds were analyzed by LC-MS^2, detection and identification of reference solutions was allowed down to 0.10 ng/mL (*i.e.* 0.1 ng, injected). At lower concentration, MS^2 spectra did not permit unambiguous compounds annotation.

2.3.4. Untargeted LC-MS^2

Analyses were performed by using ion trap full scan MS through EMS (Enhanced Mass Spectrometry) followed by an MS^2 (Enhanced Product Ion) scan triggered by IDA (Information Dependent Acquisition). MS Range used for EMS and MS^2 experiments were *m*/*z* 100-1000. IDA properties were set to select 1 to 2 peaks above 30.000 counts, and with an exclusion rule after 10 occurrences for 30 sec with dynamic background subtraction.

2.3.5. Targeted LC-MS^2

Detection of standard compounds was performed by using MRM mode followed by an MS^2 scan (MRM-MS^2) triggered by IDA filter. For this purpose, compound-specific parameters of all reference compounds were optimized using the automated compound optimization function of Analyst software by flow injection analysis (FIA) into the source: DP, EP, CE, CXP (collision cell exit potential) (Table **2**). Retention time of reference compounds could be determined by untargeted LC-MS^2 analysis of reference sample in chromatographic conditions described above. MS^2 spectra observed by FIA and MRM-MS^2 were recorded in Analyst spectral library. MRM-MS^2 parameters were set as follow, for MRM experiment: detection window (180 sec), Q1 resolution (unit), Q3 resolution (low), target scan time (2.3 sec); MS^2 scans were acquired at *m*/*z* 100-1.000. IDA properties were set to select 1 to 2 peaks above 300 counts with an exclusion filter after 10 occurrences for 30 sec with dynamic background subtraction. All reference standards compounds could be detected in MRM-MS^2 by injection of 10 μL at 10.0 ng/mL with a S/N (signal/noise ratio) above 25. Compound identification was allowed by comparison of retention time, observation of characteristic transition (S/N > 10) and by matching MS^2 spectrum of the reference compounds using Analyst library.

2.4. CHIKV virus-cell-based antiviral assay

Throughout the experiments, Vero (African green monkey kidney) cells were used. Chikungunya virus (Indian Ocean strain 899), kindly provided by C. Drosten (Institute of Virology, University of Bonn, Germany), was used. Serial dilutions of the test compounds, as well as the reference

compounds, chloroquine, were prepared in 100 µL of assay medium [MEM Rega3 (cat. no. 19993013; Invitrogen), 2% FCS (Integro), 5 mL of 200 mM L-glutamine, and 5 mL of 7.5% sodium bicarbonate], added to empty wells of a 96-well microtiter plate (Falcon, BD). Subsequently, 50 μL of a 4× virus dilution in assay medium was added, followed by 50 μL of a cell suspension. This suspension, with a cell density of 25.000 cells/50 μL, was prepared from a Vero cell line subcultured in cell growth medium (MEM Rega3, supplemented with 10% FCS, 5 mL of L-glutamine, and 5 mL of sodium bicarbonate) at a ratio of 1:4 and grown for seven days in 150 cm^2 tissue culture flasks (Techno Plastic Products). The assay plates were returned to the incubator for 6−7 days (37 °C, 5% CO_2, 95−99% relative humidity), a time at which maximal virus-induced cell death or cytopathic effect (CPE) is observed in untreated, infected controls.

Subsequently, the assay medium was aspirated, replaced with 75 μL of a 5% MTS (Promega) solution in phenol red-free medium, and incubated for 1.5 h. Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan), with the optical densities (OD values) reaching 0.6−0.8 for the untreated, uninfected controls. Raw data were converted to percentages of controls, and the EC_{50} (50% effective concentration, or concentration calculated to inhibit virus-induced cell death by 50%) and CC_{50} (50% antimetabolic concentration, or concentration that is calculated to inhibit the overall cell metabolism by 50%) values were derived from the dose−response curves. All assay conditions producing an antiviral effect that exceeded 50% were checked microscopically for signs of a cytopathic effect or adverse effects on the host cell (i.e., altered cell or monolayer morphology). A sample was considered to elicit a selective antiviral effect on virus replication only when, following microscopic quality control, at least at one concentration no CPE or any adverse effect was observed (image resembling untreated, uninfected cells). Multiple, independent experiments were performed. The antiviral experiments have been performed in a biosafety screening facility that has been validated for handling of chikungunya virus as well as the manipulation of molecules of unknown chemical safety risk. All studies have been performed by trained staff.

3. Results and discussion

3.1. Evaluation of Euphorbia extracts for selective inhibition of CHIKV replication

A total of 45 extracts were prepared from different parts of eleven Euphorbiaceae species growing wild on Corsica Island (Table 1). These extracts were evaluated for selective anti-CHIKV activity in a virus-cell-based assay (Table 2 and Table 3). As shown in Table 2, EtOAc extracts of latex from nine *Euphorbia* species exhibited a significant and selective antiviral effect on CHIKV replication (EC_{50}s <

2.1 μg/ml and SI > 31). Whereas, beside the extract prepared from *E. dendroides* latex, no aqueous extracts showed any potent anti-CHIKV activity (EC_{50} > 100 μg/ml).

Table 1

Taxonomic classification of 11 Euphorbiaceae species, and their place of harvesting in Corsica

subgenus	section	species	distribution area[a]	altitude (location)	voucher reference
Chamaesyce		*Euphobia maculata* L.	Nat. America	70 m (Palasca)	LF-022
Esula	*Aphyllis*	*Euphorbia biumbellata* Poir.	W-Stenomed	240 m (Ota)	LF-014
Esula	*Aphyllis*	*Euphorbia dendroides* L.	W-Stenomed	30 m (Piana)	LF-018
Esula	*Conicocarpae*	*Euphorbia pithyusa* L. ssp. *pithyusa*	W-Stenomed	15 m (Rogliano)	LF-004
Esula	*Helioscopia*	*Euphorbia hyberna* L. ssp. *insularis* (Boiss.) Briq.	Corsica, Sardinia	1200 m (Albertacce)	LF-013
Esula	*Helioscopia*	*Euphorbia spinosa* L.	N-Eurymed	750m (Sermano)	LF-019
Esula	*Paralias*	*Euphorbia segetalis* L. ssp. *pinea* (Hayek)	W-Stenomed	100 m (Rogliano)	LF-010
Esula	*Patellares*	*Euphorbia amygdaloides* L. ssp. *semiperfoliata* (Viv.)	Corsica, Sardinia	1300 m (Albertacce)	LF-015
Esula	*Patellares*	*Euphorbia characias* L. ssp. *characias*	Stenomed	500 m (Corte)	LF-001
Esula	*Peplus*	*Euphorbia peplus* L.	Eurosiberian	50 m (Rogliano)	LF-012
		Mercurialis annua L.	Eur. N-Africa	150 m (Rogliano)	LF-009

[a] Nat. America: Naturalized America;

W-Stenomed: Stenomediterranean from Spain to Liguria and Tunisia;

N-Stenomed: Stenomediterranean from Spain to Greece;

N-Eurymed: Eurymediterranean from Spain to Greece;

Stenomed: mediterranaen periphery;

Eur. N-Africa: Eurasia and North Africa.

Table 2

Anti-CHIKV activity of 15 extracts from 9 *Euphorbia* latex (EC_{50} and CC_{50} in µg/mL)[b]

species	plant parts	extracts[a]	CHIKV (EC_{50})	Vero cells (CC_{50})	SI[c]
E. maculata	Latex	LLE EtOAc	< 0.8	> 100	> 128
		LLE H_2O	> 100	n.d.	n.d.
E. biumbellata	Latex	SLE EtOAc	2.1 ± 1.4	> 100	> 47
E. dendroides	Latex	LLE EtOAc	< 0.8	25.0 ± 5.2	> 32
		LLE H_2O	1.1	70.7 ± n.d.	59
E. pithyusa ssp. *pithyusa*	Latex	LLE EtOAc	< 0.1	10.6 ± 2.9	> 75
		LLE H_2O	30.7	> 100	> 3.3
E. hyberna ssp. *insularis*	Latex	LLE EtOAc	< 0.8	23.8 ± 7.3	> 31
		LLE H_2O	> 100	n.d.	n.d.
E. spinosa	Latex	SLE EtOAc	< 0.8	25.9 ± 4.8	> 32
E. amygdaloides ssp. semiperfoliata	Latex	SLE EtOAc	< 0.8	34.3 ± 1.8	> 44
E. characias ssp. *characias*	Latex	LLE EtOAc	0.4 ± 0.2	16.8 ± 5.0	39
		LLE H_2O	> 100	n.d.	n.d.
E. peplus	Latex	LLE EtOAc	< 0.8	50.3 ± 14.9	> 65
		LLE H_2O	> 100	n.d.	n.d.
chloroquine (positive control)			10 ± 5 μM	n.d.	9

[a] SLE: solid-liquid extraction, LLE: liquid-liquid extraction.

[b] EC_{50} : 50% effective concentration, CC_{50} : 50% anti-metabolic concentration. Values are the median ± median absolute deviation calculated from at least 3 independent assays.

For each *Euphorbia* species, the EtOAc latex extract displayed higher anti-CHIKV activity than the EtOAc extracts obtained from plant parts (Table 3). Furthermore, EtOAc extracts of all plant parts from eight *Euphorbia* species showed potent anti-CHIKV activity (EC_{50}s < 6.9 μg/ml and SI > 5) whereas the EtOAc and MeOH extracts of *Mercurialis annua* showed weak and no anti-CHIKV activity (EC_{50} = 40 ± 4, and > 100 μg/ml, respectively). Moreover, the comparison of results obtained for various plant parts (whole plant, aerial parts, leaves, stems, roots) of *Euphobia* species highlighted that the EtOAc extracts were systematically more active than subsequent MeOH extracts. For instance, the EtOAc extract of *E. pithyusa* ssp. *pithyusa* leaves exhibited more potent anti- CHIKV activity (EC_{50} < 0.8 µg/ml, SI > 24) than the corresponding MeOH extract (EC_{50} = 23 µg/ml, SI = 1).

Based on selectivity indices (SI) and on systematic microscopic inspection of the antiviral assays, it was confirmed that the antiviral activities found for the *Euphorbia* extracts were selective. Indeed, no significant alteration of cell or monolayer morphology was observed in comparison with the untreated uninfected controls.

Table 3

Anti-CHIKV activity of 3**0** MeOH and EtOAc extracts of 8 *Euphorbia* species and *Mercurialis annua* (EC_{50} and CC_{50} in µg/mL)[c]

species	plant parts	extracts[a]	yield (%)[b]	CHIKV (EC_{50})	Vero cells (CC_{50})	SI[d]
E. biumbellata	Whole plant	ASE EtOAc	4.4	4.1	> 100	> 47
		ASE MeOH	6.7	> 100	n.d.	n.d.
E. pithyusa ssp. *pithyusa*	Leaves	ASE EtOAc	6.0	< 0.8	18.8 ± 1.2	> 24
		ASE MeOH	11.0	23.0	23.3 ± 2.8	1
	Stems	ASE EtOAc	4.6	< 0.8	15.2 ± 5.9	> 20
		ASE MeOH	9.0	4.5	8.5 ± 0.4	2
	Roots	ASE EtOAc	4.0	< 0.8	11.5 ± 1.6	> 15
		ASE MeOH	8.0	< 0.8	21.0 ± 4.5	> 27
E. hyberna ssp. *insularis*	Aerial parts	ASE EtOAc	3.9	1.0	35.5 ± 3.1	36.0
E. spinosa	Leaves	ASE EtOAc	1.0	4.8	30.9 ± 2.2	6.4
	Stems	ASE EtOAc	5.0	3.4	23.2 ± 8.2	6.8
		ASE MeOH	8.7	20.3	33.0 ± 2.4	1.6
	Roots	ASE EtOAc	7.0	< 0.8	30.5 ± 2.4	> 39.1
		ASE MeOH	4.8	2.3	54.1 ± 18.0	23.5
E. segetalis ssp. *pinea*	Aerial parts	ASE EtOAc	7.0	3.7	35.6 ± 1.6	9.6
		ASE MeOH	9.0	35.9	89.1 ± 5.1	2.5
	Stems	ASE EtOAc	3.6	3.5	25.8 ± 3.5	7.5
		ASE MeOH	5.5	57.0	> 100	> 1.7
	Roots	ASE EtOAc	4.3	1.8	30.6 ± 0.2	17.3
		ASE MeOH	5.9	29.7	> 100	> 3.4
E. amygdaloides ssp *semiperfoliata*	Whole plant	ASE EtOAc	5.2	< 0.8	30.5 ± 2.4	> 39.1
		ASE MeOH	8.0	30.6	> 100	> 3.3
E. characias ssp. *characias*	Leaves	ASE EtOAc	6.3	6.9	34.0 ± 0.8	5.0
		ASE MeOH	8.3	8.3	70.3 ± 3.4	8.5
	Stems	ASE EtOAc	5.0	2.9	32.1 ± 0.3	11.2
		ASE MeOH	3.4	8.3	70.7 ± 8.5	8.5
E. peplus	Whole plant	ASE EtOAc	7.4	4.3	63.1 ± 7.0	14.7
		ASE MeOH	7.3	30.0	> 100	> 3.3
Mercurialis annua	Whole plant	ASE EtOAc	3.1%	40 ± 4	89.1 ± 5.4	2
		ASE MeOH	6.7%	> 100	n.d.	-
chloroquine (positive control)				10 ± 5 μM	n.d.	9

[a] SLE: solid-liquid extraction, LLE*:* liquid-liquid extraction, ASE: automatic solvent extraction.

[b] based on the dry weight of vegetal material (%); n.d.: not determined.

[c] EC_{50} : 50% effective concentration, CC_{50} : 50% anti-metabolic concentration. Values are the median ± median absolute deviation calculated from at least 3 independent assays.

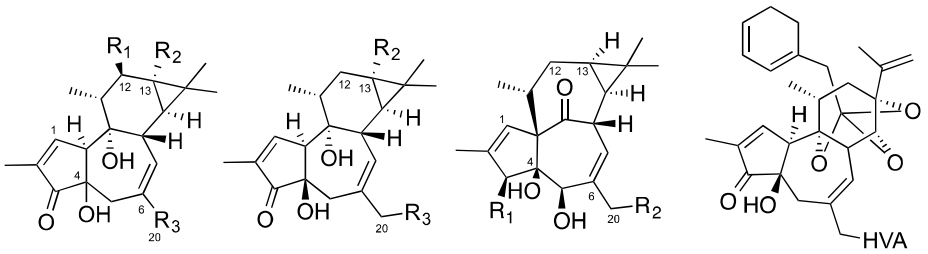
[d] SI : selectivity index (SI calculated as CC_{50}Vero/EC_{50} CHIKV).

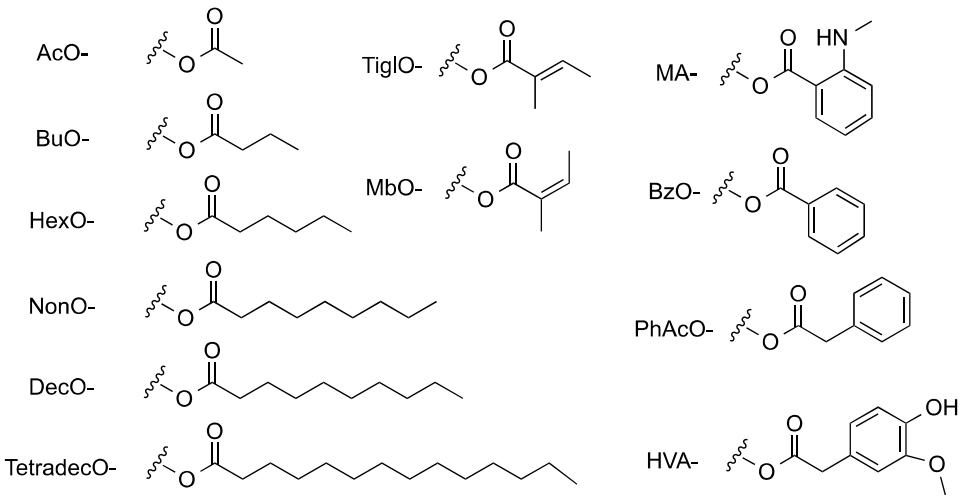
Taking into account of these results, it is likely that the anti-CHIKV activities of *Euphorbia* extracts are due to specific secondary metabolites of this genus. Previous studies have showed that *Euphorbia* extracts can exhibit antiviral [52–56], but never against CHIKV replication. Diterpenoids of tigliane- [32], ingenane- [28,33,34] and macrocyclic-type esters [35–37] isolated from various *Euphorbia* species, were found to possess potent and selective anti-HIV activities. Regarding anti-CHIKV activity of diterpenes from Euphorbiaceae, phorbol esters are the most potent inhibitors of CHIKV replication [10] but are also known for their pro-inflammatory and tumour promoter activities [13,12].

3.2. Analysis of Euphobia extracts using targeted LC-MS^2 method

In order to perform a dereplication strategy on *Euphorbia* extracts with potent anti-CHIKV activity, a LC-MS^2-based method had been developed to detect 27 commercially available natural diterpenoids (**1-27**) belonging to tigliane- (phorbol and deoxyphorbol derivatives), ingenane- and daphnane-types (Figure 1). For each standards, MS/MS optimized parameters (precursor-to-product transition monitored, declustering potential DP, entrance potential EP, collision cell entrance potential CEP, collision energy CE, collision cell exit potential CXP), and retention time in LC, had been determined and are summarized in Table 4. Main ions observed in MS^2 spectrum are also included in Table 4. The fragmentation behavior of diterpene esters were consistent with previous data reported in the literature [42,43,46,47], Indeed, in ESI positive ion mode, diterpene esters form pseudo-molecular ions, which undergo neutral loss of their acyl chain(s) under collision-induced dissociation (CID), producing the corresponding high-abundance fragment ions.

The identification of diterpenoids in plant extracts was established by comparison with reference compounds: MRM transition at specific retention time and MS^2 spectrum of the precursor ion to those recorded in the Analyst software spectral library. In addition, to avoid artefactual detection, an untargeted LC-MS^2 (MRM-MS^2) analysis was systematically carried out to confirm that the supposed targeted pseudo-molecular ion was not formed from a parent compound with a higher molecular weight.





no.	compound	R_1	R_2	R_3
1	phorbol	HO-	HO-	HO-
2	phorbol-12-acetate	AcO-	HO-	HO-
3	phorbol-12-decanoate	DecO-	HO-	HO-
4	phorbol-13-acetate	HO-	AcO-	HO-
5	phorbol-13-butyrate	HO-	BuO-	HO-
6	phorbol-13-decanoate	HO-	DecO-	HO-
7	phorbol-13-tetradecanoate	HO-	Tetradec	HO-
8	phorbol-12,13-diacetate	AcO-	AcO-	HO-
9	phorbol-12,13-dibutyrate	BuO-	BuO-	HO-
10	phorbol-12,13-dihexanoate	HO-	HO-	HO-
11	phorbol-12,13-didecanoate	DecO-	DecO-	HO-
12	4*α*-phorbol-12,13-didecanoate	DecO-	DecO-	HO-
13	12-*O*-tetradecanoylphorbol-13-acetate (TPA)	TetradecO-	AcO-	HO-
14	12-*O*-tetradecanoyl-4*α*-phorbol-13-acetate (4*α*-TPA)	TetradecO-	AcO-	HO-
15	12-*O*-tiglylphorbol-13-decanoate	TiglO-	DecO-	HO-
16	12-*O*-(*N*-methylanthranilate)phorbol-13-acetate	MA-	AcO-	HO-
17	phorbol-13,20-diacetate	HO-	AcO-	AcO-
18	phorbol-12,13,20-triacetate	AcO-	AcO-	HO-
19	20-oxo-phorbol-12,13-dibutyrate	BuO-	BuO-	=O
20	20-oxo-TPA	TetradecO-	AcO-	=O
21	12-deoxyphorbol-13-acetate (prostratin)		AcO-	HO-
22	13-*O*-isobutyryl-12-deoxyphorbol-20-acetate		BuO-	AcO-
23	13-*O*-phenylacetyl-12-deoxyphorbol-20-acetate		PhAcO-	AcO-
24	ingenol	HO-	HO-	
25	ingenol-3-mebutate	MbO-	HO-	
26	ingenol-3,20-dibenzoate	BzO-	BzO-	
27	resiniferatoxin			

Fig. 1. Structures of diterpenoids used for the targeted LC-MS^n method

Table 4

LC-MS^n parameters for reference standards in ESI positive ion mode

cpd	R_t (min)	Q1 Mass (m/z)[b]	Q3 Mass (m/z)[c]	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)	Most abundant ions (m/z) observed in MS^2 spectrum
1	1.4	387.2	369.3	156	11.5	18	21	6	369, 351, 333, 315, 311, 293
2	2.0	429.2	369.2	56	8.0	20	21	6	369, 351, 333, 329, 315, 311, 293
3	13.9	541.3	369.2	86	4.5	30	25	6	369, 351, 333, 329, 315, 311, 293
4	2.0	429.2	369.2	71	8.0	30	21	6	369, 351, 333, 329, 315, 311, 293
5	2.6	457.2	369.2	236	10	24	25	6	369, 351, 333, 329, 315, 311, 293
6	12.9	541.3	369.2	86	4.5	20	27	6	369, 351, 333, 329, 315, 311, 293
7	21.5	597.3	369.2	231	4.5	62	29	6	369, 351, 333, 329, 315, 311, 293
8	3.1	471.2	411.2	266	9.5	24	23	6	411, 351, 333, 329, 315, 311, 293
9	9.4	527.3	439.3	71	5.0	24	25	6	439, 421, 351, 333, 329, 315, 311, 293
10	17.7	583.3	467.2	301	6.5	30	25	6	467, 351, 333, 329, 315, 311, 293
11	28.5	695.5	523.3	91	9.0	50	29	8	523, 351, 333, 329, 315, 311, 293
12	29.0	695.5	523.3	211	10.5	24	45	6	523, 351, 333, 329, 315, 311, 293
13	23.5	639.4	411.2	71	8.5	44	29	6	579, 411, 351, 333, 329, 315, 311, 293
14	24.4	639.4	411.2	71	8.5	44	29	6	579, 411, 351, 333, 329, 315, 311, 293
15	22.2	623.4	523.3	206	9.0	30	27	6	523, 451, 351, 333, 329, 315, 311, 293
16	9.3	562.2	411.2	301	5.0	24	25	6	411, 502, 351, 333, 329, 315, 311, 293
17	2.8	471.2	411.2	266	9.5	24	23	6	411, 351, 333, 329, 315, 311, 293
18	5.8	513.2	453.2	66	4.5	20	25	6	453, 393, 365, 333, 315, 311, 293
19	3.5	525.2	437.2	71	6.0	20	27	8	437, 349, 331, 309, 291
20	24.0	637.3	409.2	71	8.0	27	27	6	577, 409, 349, 331, 309, 291
21	3.3	413.2	353.2	156	8.0	24	21	6	353, 335, 317, 313, 307, 295
22	11.3	483.2	395.2	71	7.0	22	25	6	335, 317, 313, 307, 295
23	11.7	531.2	395.2	116	8.0	26	27	6	335, 317, 313, 307, 295
24	3.0	371.1	353.2	261	10.0	28	23	6	353, 335, 317, 313, 307, 295, 285
25	12.4	453.2	353.2	126	7.5	20	23	6	353, 335, 317, 313, 307, 295, 285
26	18.2	579.4	457.2	96	9.0	28	27	6	457, 335, 317, 313, 307, 295, 285
27	16.3	651.3	515.2	266	10.5	30	33	6	515, 469, 441, 333

LC-MS^2 analysis of the EtOAc *Euphorbia* extracts allowed the identification of three diterpenes (Table **5**). The results indicated that ingenol-3-mebutate (**25**) was present in *E. peplus* extracts (latex and whole plant). Indeed, the identification of compound **25** was allowed by the detection of a transition *m*/*z* 453→ 335 at R_t 12.4 min and by comparison with MS^2 spectra recorded in our spectral library (Fig. S1). Using the same methodology, ingenol-3,20-dibenzoate (**26**) was detected by observation of a transisiton *m*/*z* 579→457 at R_t 18.3 min in *E. peplus* and *E. segetalis* ssp. *pinea* extracts (Fig S2-S3). Compound **26** was found in latex extracts as well as in other plant parts of the two species. Furthermore, 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate (**22**) was detected in roots and aerial parts extracts of *E. segetalis* ssp. *pinea* (Fig S4).

Table 5

Reference standard diterpene esters detected by targeted LC-MS (MRM-MS^2) (see Fig. S1-S4 for MS^2 spectra)

extracts (EtOAc)	plant parts[a]	compound	transition	R_t (min)	$[M+Na]^+$ (*m/z*)	ions observed in MS^2 spectrum	S/N
E. peplus	Lat, WP	ingenol-3,20-dibenzoate **26**	579.4/457.2	18.3	579.21	457.2, 335.2	188
		ingenol-3-mebutate **25**	453.2/353.2	12.4	453.22	335.2, 295.2	82
E. pithyusa ssp. *pithyusa*	Lat, OPP	13-*O*-isobutyryl-12-deoxyphorbol-20-acetate **22**	483.2/395.2	11.2	483.24	423.4, 395.2, 335.2	85
E. segetalis ssp. *pinea*	R, AP	ingenol-3,20-dibenzoate **26**	579.4/457.2	18.4	579.21	457.2, 335.2	145

[a] Lat : latex, WP : whole plant, OPP : other plant parts, R : roots, AP : aerial parts

The present results are in agreeement with the previously reported isolation of ingenol-3-mebutate (**25**) in *E. peplus* extract [57], and the isolation of other ingenane-type esters in *E. peplus* and *in E. segetalis* extracts [58,59]. The detection of 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate (**22**) is also reinforced by the isolation of deoxyphorbol diesters in *E. pithyusa* ssp. *pithyusa* [60]. However, one must keep in mind that even if comparison with MS^2 spectrum and R_t obtain in the same conditions is considered sufficient to confidently identified compound in LC-MS^2 [61], it cannot be exclude that the ion detected is an isomer.

Beside detection of these three compounds, no other standard compound could be detected in the *Euphorbia* extracts, including the highly potent anti-CHIKV phorbol esters **11**, **13**, and **21**. These results are consistent with (i) previous phytochemical investigation of these species (Table S1) and (ii) the fact that, while tigliane-type diterpene are commonly found in *Euphorbia* spp. [27,31], occurrence

of phorbol derivatives *stricto sensu* was rare in this genus [13,62,63]. Indeed, most of phorbol esters had been found in genus *Croton* [64], *Jatropha* [65] and *Sapium* [66].

From the dereplication perspective, taking into account the IC_{50} values for inhibition of CHIKV replication of 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate (**22**), and ingenol-3,20-dibenzoate (**26**), (EC_{50} = 0.7 ± 0.1 μM, SI = 5.0, and EC_{50} = 1.2 ± 0.1 μM, SI = 6.4, respectively), it can be concluded that the presence of compound **22** in *E. pithyusa* ssp. *pithyusa*, and compound **26** in *E. peplus* and *E. segetalis* ssp. *pinea* extracts should partially explain their potent anti-CHIKV activity. However, it is likely that other diterpene esters may contribute to the anti-CHIKV activities of these extracts. For instance, it has been demonstrated that jatrophane esters isolated from *E. amygdaloides* ssp. *semiperfoliata* exhibited significant anti-CHIKV activities [51].

It should be noted that the diterpenes **22**, **25**, and **26** are potent and selective inhibitors of HIV-1 and HIV-2 at the nanomolar scale [10]. Thus, it is likely that the EtOAc extracts of *E. peplus*, *E. segetalis* ssp. *pinea* and *E. pithyusa* ssp. *pithyusa* possess strong anti-HIV activities.

4. Conclusion

In conclusion, several EtOAc and MeOH extracts of *Euphorbia* species exhibited potent and selective inhibitory activities of CHIKV replication. A LC-MS^2 based method was used to detect the possible presence in the biologically active extracts of diterpene esters endowed with antiviral activities. The results of the LC-MS^2 analysis indicated the presence of 13-*O*-isobutyryl-12-deoxyphorbol-20-acetate (**22**) in *E. pithyusa* ssp. *pithyusa* extracts, ingenol-3-mebutate (**25**) in *E. peplus* extracts, ingenol-3,20-dibenzoate (**26**) in *E. peplus* and *E. segetalis* ssp. *pinea* extracts. Potent inhibitors of CHIKV replication such as phorbol-12,13-didecanoate (**11**)**,** TPA (**13**) and prostratin (**21**) were not detected in the *Euphorbia* extracts. Thus, the present results suggested that untargeted diterpene esters are responsible of the anti-CHIKV activity of *Euphorbia* extracts.

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